for certain proportions of β-form and disordered coil. Here the fit in the 230 region is better with the constants of the sodium dodecyl sulfate-produced β-form.

We are deeply indebted to Prof. J. T. Yang and Drs. E. S. Pysh and P. Urnes for numerous helpful and critical discussions and aid with the circular dichroic work.

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† On leave from the Cardiovascular Research Institute, University of California Medical Center, San Francisco, California.

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*THE EFFECT OF HORMONES ON DNA**

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Gene activation and repression have been considerably elucidated in bacteria.[1, 2] Comparable mechanisms, although of greater complexity, probably exist in the cells of higher organisms. Hormones seem to be involved in the latter, for structural genes are in many cases activated by hormones.[3] Hormonal action may take

place at more than one level, but certain evidence suggests that one of the levels is chromosomal. Autographic evidence reveals the localization of administered testosterone[4] and aldosterone[5] within chromosomes, and microscopic observation shows that specific areas of certain chromosomes can be puffed by the juvenile hormone ecdysone and that the puffing is associated with increased synthesis of messenger RNA.[6] Puffing implies that the physical state of the chromosome has been changed by hormone activation, and this concept is corroborated by the discovery that lymphocyte heterochromatin, which is inactive in RNA synthesis, is relatively densely packed, whereas lymphocyte euchromatin, which is active in RNA synthesis, is relatively loosely packed.[7]

If hormones cause a loosening of chromosomal material, this effect may be attributable in part to events on a molecular level, in which the complementary strands of the DNA double helix are separated as a result of weakening of the intrastrand linkages. The absorbance of DNA at 260 mμ is a sensitive indicator of the degree of separation of the strands by various chemical and physical agents,[8] and provides a means of studying the effect of hormones on the intrastrand linkages. Accordingly, we have examined the effect of physiological concentrations of hormones on the separation of DNA strands by heat. It was assumed that a given hormone would act only on a short, specific nucleotide sequence rather than on the intrastrand bonds as a whole, in which case each hormone would probably affect only a portion of the melting curve.

In the experiments to be described, we will show reproducible alterations in the melting profile of DNA in the presence of representatives of every class of hormone (steroid, protein, and amino acid derivatives) at concentrations well within the physiological range.

Materials and Methods.—Reagents: All chemicals were of reagent grade. Only double-distilled water was used. As a general rule, all substances were dissolved in sodium phosphate buffer, 7×10^{-4} *M*,[9] at the appropriate pH. The term phosphate buffer as used in this report refers to this solvent.

Hormones: Steroid hormones and L-thyroxine were obtained from Sigma Chemical Co., St. Louis. Porcine insulin (Lilly Research Laboratories), a recrystallized, trypsin-treated material containing 0.51% zinc and less than 0.005% glucagon was used; the insulin activity of the preparation was approximately 24 units/mg. Crystalline human somatotropin was prepared by C. H. Li; samples were inactivated as needed by boiling for 15 min at a concentration of 0.2 μg/ml in phosphate buffer, pH 6.9. L-epinephrine (USP) was obtained from DesMo Chemical Co., New York.

The steroid hormones were dissolved with the aid of acetone, which was then evaporated from the solution by boiling. The concentration of steroids was assayed spectrophotometrically.[10] The solutions were kept at room temperature because of their low solubility. Insulin was stored as an acidic stock solution because of its poor solubility at pH 6.9; dilutions in the phosphate buffer were made up at the proper pH just before use.

DNA and deoxyribonucleoprotein: DNA was prepared from *B. subtilis* and human placental nuclei essentially as described by Marmur.[11] The isolation of placental nuclei and more complete studies of DNA isolated from these nuclei will be described in a subsequent publication.[12] The DNA was stored at 4°C in 0.15 *M* sodium chloride–0.015 *M* sodium citrate, pH 7.65, in tubes containing a drop of chloroform. DNA not stored with chloroform was equally active. Poly d(A·T), poly d(G·C), and infectious λ-phage DNA were supplied by Dr. Michael J. Chamberlin.

DNA was denatured by maintaining a solution of DNA in phosphate buffer at 100°C for 10 min, then cooling it rapidly. DNA was sheared at a concentration of 130 μg/ml in phosphate buffer in a Virtis homogenizer run at top speed for 20 min. The cup containing the DNA was packed in ice during this time.

Deoxyribonucleoprotein was prepared from placental nuclei by extraction in phosphate buffer, followed by centrifugation for 30 min at 56,000 × *g*.[12]

Glassware: Since the hormones tested were active at extremely low concentrations, great care was taken to prevent contamination of glassware and solutions. The glassware was routinely soaked in acid-dichromate cleaning solution and then rinsed in hot alkaline detergent before each use.

Melting temperature technique: The hyperchromicity of DNA caused by heating was studied as described by Marmur and Doty.[8] The solvent used in all experiments was 7×10^{-4} *M* phosphate buffer. The temperatures recorded were those of the heating bath (except in the experiment shown in Fig. 1, where a thermister was used to measure the temperature in a blank cuvette). Reaction mixtures were first incubated at 25°C for 15 min; the temperature in the circulating water-bath was then raised in increments of 2–3°C. The system was maintained at a given temperature for 10 min before each series of absorbancy readings. After every 12°C rise in temperature, the reaction mixtures were stirred. Only glass stirring rods were used, because steroids may be soluble in plastics. Care was taken that all the cuvettes in an experiment were at the same pH and ionic strength.

Results.—The experimental results are shown in Figures 1 through 12. The data summarized in any one figure were obtained during the same experiment. All the results presented were reproduced in several experiments. The curves obtained in replicate experiments were similar but not identical, probably due to unavoidable differences in the rate of heating and to minor discrepancies in the concentration of reactants.

Effect of cation: The substitution of potassium for sodium did not affect the results of these experiments.

Effect of steroid hormones: The majority of the experiments were conducted with purified placental DNA. A typical melting curve for this preparation is shown in

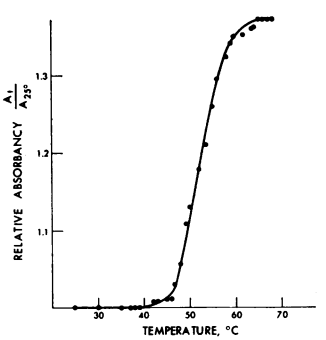

FIG. 1.—Melting curve of placental DNA. DNA was heated in sodium phosphate buffer, 7×10^{-4} *M*, pH 7.65. Sample was read against phosphate buffer as a blank.

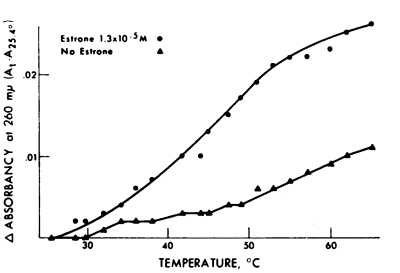

FIG. 2.—Absorbancy changes in DNA (16 μg/ml) heated at pH 7.7 with and without estrone. Phosphate buffer or phosphate buffer and estrone (as appropriate) were used as the blanks.

Figure 1. Preliminary tests showed that no change in absorbancy resulted from heating any of the hormones in solutions without added DNA.

The effect of estrone on DNA is depicted in Figure 2. In this experiment DNA was heated with and without estrone at a hormone concentration of 1.3×10^{-5} *M*, and both reactant mixtures were read against a buffer blank. As shown, the absorbancy of the DNA increased more rapidly in the presence of estrone than in its absence. The difference was noted only in the early portions of the melting curves.

Since this was true in general of all the experiments, most of the studies were terminated at relatively low temperatures.

The effect on DNA of 17β-estradiol at varying concentrations is shown in Figure 3. In this and subsequent figures the ordinate shows the absolute difference, A_h–A_0, between the absorbancy, A_h, of the DNA-hormone mixture and the absorbancy, A_0, of the DNA alone at the temperatures indicated. The value A_h–A_0 was independent of DNA concentration in the studied range between 1.5 and 17.3 μg/ml. The curves in Figure 3 clearly show that the effect of the estradiol was a function of the hormone concentration. An interesting feature is the sharp jump in absorbancy between 35°C and 38°C at the highest concentration of estradiol. This same discontinuous function was seen later in studies of cortisol (*vide infra*).

17β-Estradiol is estrogenic *in vivo*, whereas 17α-estradiol is not. This pair of isomers provides a nice means of testing the ability of DNA to discriminate between closely related molecules. Figure 4 compares the effects of 17β-estradiol and 17α-estradiol on DNA. As shown, 17β-estradiol exerted a substantial influence on the melting behavior of DNA, whereas 17α-estradiol had no effect alone and indeed inhibited the action of 17β-estradiol when both were present in the reaction mixture. These effects of the steroids resemble their known biological activity in a remarkable fashion. The importance of the 17 position is also manifested by testosterone, for 17β-testosterone is biologically active while 17α-testosterone is not. The insolubility of testosterone, however, precludes its being tested in this system.

The effect of cortisol on DNA was similar to that of estrogens. Figure 5 shows the action of different concentrations of cortisol on DNA; a break in the curve occurred at the highest concentration, comparable to that seen in the study of β-estradiol and DNA (Fig. 3).

Effect of protein hormones: The action of insulin on the melting of DNA was also examined. A definite effect was found (Fig. 6), although the shapes of the curves differ considerably from those obtained in experiments with steroid hormones. The most effective concentration, 6.9×10^{-10} M, was that commonly found in normal human serum.

Somatotropin, another protein hormone, also affected the melting of DNA (Fig.

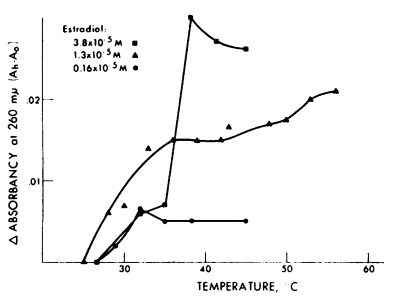

FIG. 3.—Absorbancy changes in DNA (17 μg/ml) heated at pH 6.9 with various concentrations of 17β-estradiol. In this and subsequent figures, the ordinate shows the absolute difference, $A_h - A_0$, between the absorbancy, A_h, of the DNA-hormone mixture and the absorbancy, A_0, of DNA alone at the indicated temperatures.

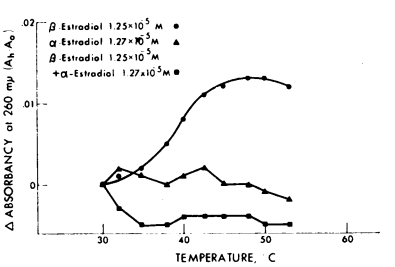

FIG. 4.—Comparison of the action on DNA of 17β-estradiol and 17α-estradiol. DNA (13.6 μg/ml) was heated at pH 7.6 with the indicated isomer of estradiol.

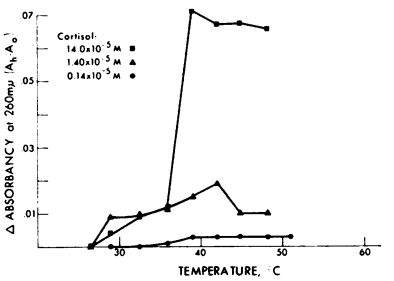

FIG. 5.—Absorbancy changes in DNA (17 μg/ml) heated at pH 6.9 with various concentrations of cortisol.

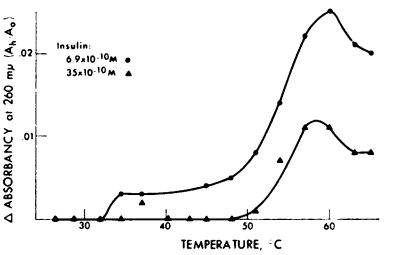

FIG. 6.—Absorbancy changes in DNA (17 μg/ml) heated at pH 6.9 with various concentrations of insulin. Insulin at a concentration of 350 × 10^{-10} M was completely inactive.

7). When the somatotropin was boiled first for 15 min (which destroys the hormone's biological activity), it no longer had any significant effect on the DNA. Before boiling, somatotropin was active at 10^{-13} M, approximately the concentration found in serum.

Insulin and somatotropin are well-known antagonists in biological test systems, and the antagonism was apparent in their combined effect on the melting of DNA, as shown in Figure 7.

Effect of hormones derived from amino acids: L-epinephrine had a substantial effect on the melting of DNA at hormone concentrations of about 1 × 10^{-11} M, which is below commonly accepted values for tissue levels (Fig. 8). Higher concentrations proved to be inhibitory.

L-thyroxine, which is present in tissues in concentrations of about 3 × 10^{-6} to 7 × 10^{-6} M, had no effect on the melting of DNA in concentrations ranging from 5.6 × 10^{-6} to 2.3 × 10^{-4} M. The failure of nuclear DNA to respond to thyroxine is not surprising, for thyroxine may act only on the mitochondrial DNA.[13] For this reason, studies now in progress on the action of thyroxine on mitochondrial DNA will be of interest.

Effect of estrone on deoxyribonucleoprotein: The effect of estrone on deoxyribo-

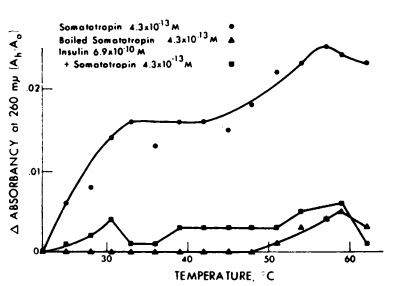

FIG. 7.—Absorbancy changes in DNA (17 μg/ml) heated at pH 6.9 with somatotropin and with somatotropin and insulin.

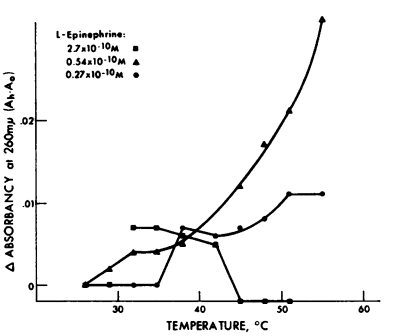

FIG. 8.—Absorbancy changes in DNA (17 μg/ml) heated at pH 6.9 with the indicated amounts of L-epinephrine. L-epinephrine at concentrations of 11 × 10^{-10} M was completely inactive.

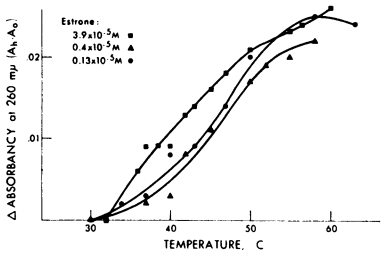

FIG. 9.—Absorbancy changes in deoxyribonucleoprotein (DNP) (18.3 μg of DNA/ml) heated at pH 7.7 with the indicated amounts of estrone.

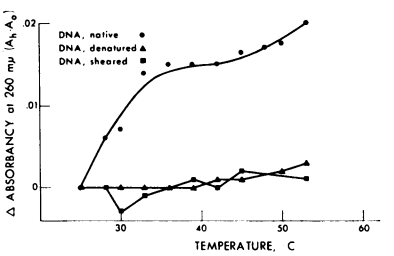

FIG. 10.—Effect of the physical state of DNA on the change in absorbancy of DNA (17 μg/ml) heated at pH 6.9 with 1.3×10^{-5} *M* estradiol.

nucleoprotein was similar to its action on DNA (Fig. 9). This finding is perhaps surprising, for the protein moiety of the nucleoprotein complex stabilized its melting behavior. The T_m of the deoxyribonucleoprotein in this study, for instance, was 79°C, compared with a T_m of 52°C for the pure DNA.[12]

Effect of estradiol on nonplacental DNA: Estradiol had only a slight effect on DNA prepared from *B. subtilis*. This lack of response could reflect some unknown and nonspecific alteration in the physical state of the sample of bacterial DNA, or it could represent a more specific inability of bacterial DNA to respond to mammalian hormones. It would be of interest to see the effect on human DNA of a hormone from a distant species, for example, ecdysone from insects.

Infectious (and therefore relatively intact) λ-phage DNA and the synthetic copolymers, poly d(A·T) and poly d(G·C), were also tested with the hormones estradiol, cortisol, and insulin. The hormones had no apparent influence on the melting temperature of these samples of DNA. This observation is consistent with the concept that the hormones react only with specific sequences of nucleotide linkages.

Miscellaneous factors influencing the assay system: DNA is ordinarily considered to be a stable molecule, but there were unpredictable variations in the suitability of certain preparations as indicators of hormone action. We have found that to be suitable as an indicator the DNA must be of sufficiently high molecular weight to form typical silky strands after the addition of 2 vol of 90 per cent ethanol. Satisfaction of this requirement, however, is not always enough, for the simple process of pipetting numerous samples of DNA from a single tube of solution for a series of experiments appeared to diminish the reactivity of the DNA without impairing its alcohol insolubility. Age alone does not inactivate DNA, for preparations over a year old have shown satisfactory responses to hormones if they were stored as described in the section on *Methods*.

When a responsive preparation of DNA was deliberately denatured by heat or degraded by shear, the material became unresponsive to hormone action (Fig. 10). The action of shearing is of particular importance since it does not affect the melting point of DNA.[8]

Occasionally an inactive DNA preparation could be made responsive by heating at 35°C, as shown by the example in Figure 11. Essential to this effect was the addition before heating of a small drop of chloroform to the solution of DNA, although chloroform in the absence of heat was unable to activate the DNA.

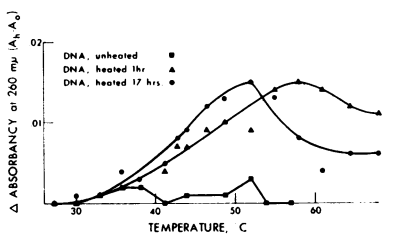

FIG. 11.—Effect of preincubation on the absorbancy changes in DNA heated in the presence of estradiol. DNA (247 μg/ml) was incubated at 35°C (pH 7.7) for the length of time indicated and was allowed to return to room temperature. The DNA (16.5 μg/ml) was then reheated at pH 7.7 with 1.25×10^{-5} *M* estradiol.

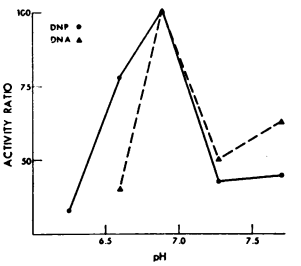

FIG. 12.—Effect of pH on the changes in absorbancy when DNA (19.5 μg/ml) and deoxyribonucleoprotein (DNP) (equivalent to 49.3 μg of DNA/ml) were heated at the indicated pH with 1.3×10^{-5} *M* estradiol. The activity ratio indicates the maximum increase in absorbancy relative to the absorbancy at pH 6.9, which was set equal to 100.

The pH optimum was found to be pH 6.9, whether DNA or deoxyribonucleoprotein was used as the indicator (Fig. 12).

A puzzling obstacle to the establishment of a rapid assay was the lack of difference in absorbancy between solutions with and without hormone when they were raised rapidly to a temperature which if attained by slow heating would reveal a marked difference in absorbancy. Repeated efforts to circumvent this problem were unsuccessful.

Discussion.—We have shown that three types of hormones—steroid, protein, and amino acid derivatives—are able to destabilize portions of the DNA molecule as assessed by thermal denaturation. The activity of the hormones is high when they are in concentrations similar to those found in the intact organism and reflects exquisite degrees of specificity, e.g., β-estradiol is active and α-estradiol is inactive. Other resemblances to the known biological behavior of hormones were noted: boiling somatotropin destroyed its effect on DNA, insulin and somatotropin were mutually antagonistic, and L-thyroxine, which probably does not act on nuclear genes,[13] had no effect on nuclear DNA.

A number of organic substances have been shown[14, 15] to affect the melting temperature of DNA, but enormously greater concentrations than those used in this study are required and the effect is generally nonspecific. Such effects are thought to result from the stabilization of denatured DNA by decreasing the ion solvating properties of the solvent and increasing its hydrophobic properties.

It is reasonable to postulate that the biological action of certain hormones is directly on the DNA molecule itself, and that this action consists of a destabilization of intrastrand linkages in specific segments of the macromolecule, resulting in a separation of strands prior to transcription. This proposal is consistent with the observations by Frenster *et al.*[7] and Littau *et al.*[16] that active chromatin is more diffuse and extended than inactive (repressed) chromatin. An alternative explanation is that the hormone action was mediated by an undetected component. The sensitivity of the assay system used was such that if this component were protein, no more than 0.2 μg/ml could have been present in the reaction mixture.

We could find no difference between deoxyribonucleoprotein and DNA in their

reaction to hormones in our limited series of experiments. In his studies, Frenster[17] found that inactive chromatin showed a greater increase in absorbancy on heating than active chromatin. The same was true of DNA isolated from inactive and active chromatin, except that the thermal hyperchromicity of inactive and active chromatin differed to a greater extent. We interpret these results as indicating that the protein in active chromatin either helps to pull the strands apart or aids in maintaining DNA as an open structure.

Summary.—DNA from human placental nuclei was incubated with each of the following hormones (all at physiological serum concentrations): estrone, 17β-estradiol, cortisol, insulin, somatotropin, and epinephrine. Alteration of the melting profile of the DNA was used to assess the effect of the hormone on the strength of the DNA intrastrand bonds. All of the hormones studied caused a weakening of the bonds. 17α-Estradiol (the biologically inactive isomer) and boiled somatotropin were inactive, as was L-thyroxine. A deoxyribonucleoprotein complex isolated from the nuclei of placental cells was affected by the hormones in the same way as pure DNA. Denatured DNA, sheared DNA, and DNA isolated from *B. subtilis* were all unaffected by the hormones. Two types of synthetic DNA, poly d(A·T) and poly d(G·C), also were not affected by the hormones, implying a need for stereospecific fit between hormones and DNA.

We have concluded that one action of hormones is to activate genes by promoting the separation of complementary strands of specific segments of the DNA double helix prior to transcription.

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